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The effect of phosphate on the unfolding-refolding of phosphoglycerate kinase induced by guanidine hydrochloride

T. Chardot, A. Mitraki, Y. Amigues, M. Desmadril, J.M. Betton and J.M. Yon

Laboratoire d'Enzymologie Physico-Chimique et Moléculaire, GR-CNRS associé à l'Université de Paris-Sud, 91 405 Orsay, France

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Phosphate ions were found to stabilize the native structure of phosphoglycerate kinase without modifying the folding pathway. The transition curves obtained from different signals: enzyme activity, ellipticity at 220 nm and fluorescence intensity at 336 nm (excitation at 292 nm) are shifted to smaller guanidine hydrochloride c_m values in the absence of phosphate. The kinetic characteristics are qualitatively similar, unfolding rate constants being slightly smaller in the presence of phosphate. The mechanism by which the native structure of phosphoglycerate kinase is stabilized by phosphate probably occurs upon specific phosphate binding to the nucleotide β- or γ-phosphate binding site of nucleotides.

Protein folding; Phosphoglycerate kinase; Phosphate effect; Equilibrium; Kinetics; Structural domain

1. INTRODUCTION

The stabilization of the native conformation upon ligand binding has been described for several enzymes. In previous studies [1–4], the unfolding-refolding transition of horse muscle phosphoglycerate kinase (PGK) induced by guanidine hydrochloride (Gdn HCl) was studied in the presence of phosphate ions (100 mM phosphate buffer, pH 7.5). Under these conditions, the loss of enzyme activity was observed for very small concentrations of denaturant (0.8 M Gdn HCl), and accompanied by a decrease of about 60% of the α-helix and 25% of the β-structure content. A part of the molecule, located in the C-terminal domain which contains the four tryptophan residues, was found to be more resistant to denaturation and folded prior to the N-terminal domain. The enzyme possesses both nucleotide and phosphoglycerate binding sites, and since both nucleotides and phosphoglycerate substrates contain phosphate groups, phosphate ions are expected to bind quite specifically to the enzyme and as a consequence a modification in the stability of the enzyme could occur. In order to determine the role of phosphate ions, the transition was reinvestigated in the absence of phosphate, and the results compared with those obtained in the presence of phosphate.

Correspondence address: T. Chardot, Laboratoire d'Enzymologie Physico-Chimique et Moléculaire, GR-CNRS associé à l'Université de Paris-Sud, 91 405 Orsay, France

2. MATERIALS AND METHODS

Horse muscle phosphoglycerate kinase (EC 2.7.2.3) was prepared according to a slight modification of Scopes' procedure [5] as introduced by Blake et al. [6]. The enzyme activity was measured using Bücher's procedure [7].

Unfolding and refolding experiments were carried out either in a 20 mM Pipes buffer, pH 7.5, containing 1 mM EDTA and 10 mM 2-mercaptoethanol, or in a 20 mM Tris buffer, pH 7.5, containing 0.5 mM dithiothreitol and 1 mM EDTA.

The stock solution of PGK (10–100 μM) was diluted to a final concentration of 2 μM and incubated in the denaturant for 24 h at 23°C for unfolding. For refolding experiments, the PGK solution was incubated in Gdn HCl for 24 h at 23°C and then diluted in different concentrations of denaturant. The final PGK concentration was 2 μM.

The fluorescence intensity was measured at 336 nm with a Perkin Elmer MPF 44B spectrofluorimeter (excitation at 292 nm). Circular dichroism spectra were recorded with a Mark V Jobin and Yvon dichrograph. The temperature was kept constant at 23°C for all experiments.

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3. RESULTS

3.1. *Study of the transition induced by Gdn HCl in the absence of phosphate*

Three signals were used to follow the transition in the absence of phosphate, enzyme activity, variation in ellipticity at 220 nm and fluorescence emission intensity at 336 nm (excitation at 292 nm).

The transition curve followed by the enzyme activity was represented by a single and very cooperative transition, slightly shifted towards lower Gdn HCl concentrations when compared with the corresponding transition in the presence of phosphate (fig.1A,B and table 1).

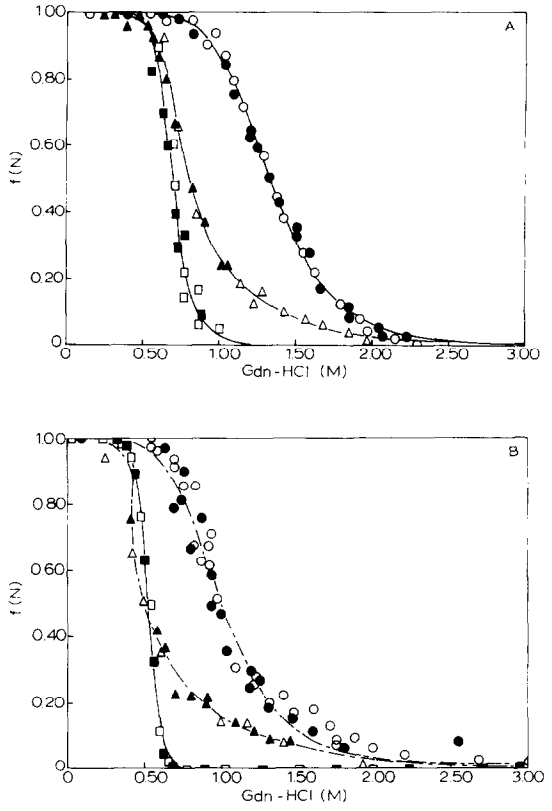

Fig.1. Gdn HCl induced folding and unfolding of phosphoglycerate kinase. Transitions curves followed by several signals: fluorescence intensity at 336 nm (excitation at 292 nm) (○,●), ellipticity at 220 nm (△,▲) and enzyme activity (□,■. (filled symbols) Unfolding process; (open symbols) refolding process. Enzyme concentration, 2 μM; all experiments were carried out at 23°C and pH 7.5. (A) In the presence of phosphate 100 mM, 1 mM EDTA, 10 mM 2-mercaptoethanol or in circular dichroism experiments 1 mM DTT; (B) in the absence of phosphate; 20 mM Pipes buffer containing 0.5 mM DTT or 10 mM 2-mercaptoethanol and 1 mM EDTA.

The same typical dichroic spectra were recorded between 200 and 260 nm in the absence and in the presence of phosphate for the native and denatured protein. By following variations in ellipticity at 220 nm an asymmetrical transition curve was obtained; it was decomposed into two transitions according to Betton et al. [2]; one which represented about 52% of the total amplitude exhibited the same c_m as the transition observed from the enzyme activity, i.e. slightly smaller than the c_m observed in the presence of phosphate (0.45 ± 0.05 M Gdn HCl). The other part of the transition with a c_m of 0.7 ± 0.05 M Gdn HCl seemed slightly smaller than the one observed by fluorescence measurements, c_m being the mid-point of each transition curve obtained for f_N = 0.5.

The total variation in fluorescence emission intensity at 336 nm was not dependent on the presence of phosphate. The transition curve obtained following this signal was found to be symmetrical but with a smaller c_m value (c_m = 0.97 ± 0.05 M Gdn HCl) than found in the presence of 100 mM phosphate (1.40 ± 0.05 M; table 1).

Table 1

Comparison of the thermodynamic parameters of the transition in the presence and absence of phosphate

Observable	n	C_m (M Gdn HCl)	$\Delta b_{2,3}$ (M^{-1})	$\Delta G°$ (kcal/mol)
In 100 mM phosphate				
enzyme activity	12 ± 3	0.7 ± 0.05	12	−6 ± 0.5
ellipticity (220 nm)	$3<n<12$	0.8 ± 0.05	14–3	−3 ± 0.5
fluorescence (336 nm)	6.5 ± 0.5	1.3 ± 0.02	5	−3 ± 0.5
In absence of phosphate				
enzyme activity	15 ± 3	0.5 ± 0.05	20	−5 ± 0.5
ellipticity (220 nm)	$2.5<n<8$	0.45–0.7	10–4.6	−3.3 ± 0.5
fluorescence (336 nm)	4.3 ± 0.5	1.0 ± 0.05	3.7	−2.4 ± 0.5

n, cooperativity index; c_m, concentration of denaturant corresponding to the mid-point; $\Delta G°$, the variation in free energy of the transition in the absence of denaturant; $\Delta b_{2,3}$ is defined in the text

3.2. *Thermodynamic analysis*

Using several empirical relationships, it was possible to determine several thermodynamic parameters. n, the cooperativity index was obtained from the equation:

$$K_{app} = K_0 c^n,$$

proposed by Tanford [8] and Schellman [9], K_{app} being the experimental equilibrium constant, K_0 the one extrapolated in the absence of denaturant and c the denaturant concentration. The free energy variation between the native and the denatured protein in the absence of denaturant can be estimated from the value of K_0 or from the following expression:

$$\Delta G_{app} = \Delta G_0 + \Sigma \alpha_i n_i \delta g_{i,tr},$$

n_i being the number of groups i, δg_i the free energy of transfer of groups i from the solvent to water; these values can be obtained for each amino acid from the table given by Nozaki and Tanford [10]. α is a parameter corresponding to the variation in accessibility of the groups to the solvent from the native to the denatured form. It can be determined from the evaluation of accessible surface area [11]. Table 1 summarizes the parameters obtained from the different signals in the absence and presence of phosphate.

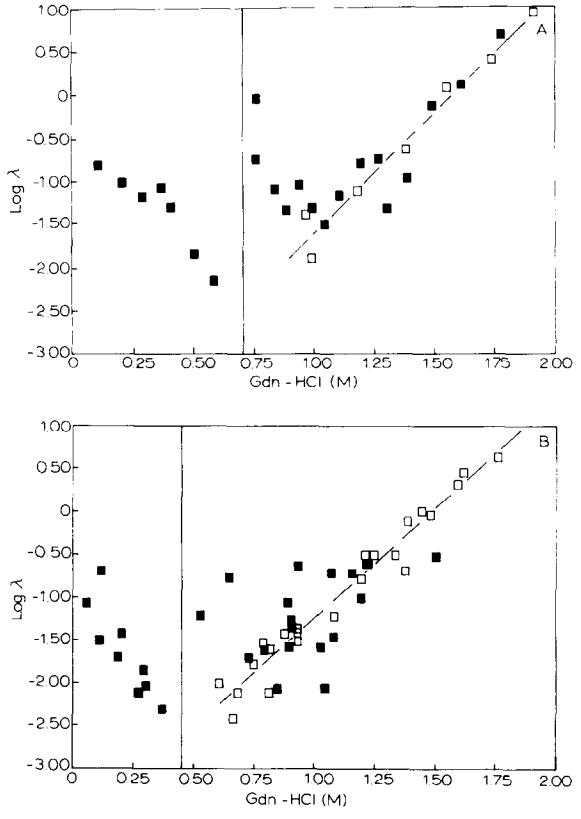

Fig.2. Variations of the macroscopic rate constants versus Gdn HCl concentration for the unfolding (■) and for the refolding (□) of phosphoglycerate kinase. (A) In the presence of phosphate; (B) in the absence of phosphate in conditions identical to those in fig.1.

3.3. *Kinetic studies of the transition in the absence of phosphate*

The kinetic behavior of the unfolding and refolding of PGK in the absence of phosphate was qualitatively similar to that observed in the presence of phosphate. The denaturation process was monophasic and faster in the absence of phosphate. For the refolding process, the macroscopic rate constants of the slow phase reached a maximum value for lower Gdn HCl concentrations in the absence than in the presence of phosphate (fig.2).

4. DISCUSSION

A comparison of the data obtained in the absence and presence of phosphate ions indicates a protection of the native structure of the enzyme in the presence of phosphate. Several regions of the molecule containing α-helices and β-strands are stabilized by phosphate. They are at least partially localized in the C-terminal domain since the transition observed from the fluorescence signal is shifted towards higher concentrations of denaturant in the presence of phosphate. A protection of the N-terminal region of the molecule cannot be excluded but there are no signals to specifically detect the conformation of this domain.

Phosphate ions stabilize the native structure of PGK without modifying the folding pathway as indicated by the similarity in the kinetic characteristics with and without phosphate. The mechanism of such a protection probably occurs upon phosphate binding to a particular site of the protein. Nucleotide phosphates (ADP, ATP) bind to the C-terminal domain and thus protect the enzyme against the denaturation induced by Gdn HCl (Mitraki, A. unpublished), whereas 3-phos-

phoglycerate does not. However the binding site of 3-phosphoglycerate is still a matter of controversy. It is assumed to be located either in the N-terminal domain [12,13] or between the two domains [14]. It is more likely that phosphate ions protect the native structure through their binding to the corresponding sites of the nucleotide phosphate substrates. A possible binding site could be the β- or the γ-phosphate of the ATP molecule located in the vicinity of α_{13}-helix. An electrostatic interaction between the dipole of the helix and the negative charges of phosphate in nucleotides is often encountered in kinases and dehydrogenases [15]. Furthermore, crystallographic studies have revealed in the yeast enzyme two binding sites for sulfate ions; these ions have some similarities with phosphate ions concerning the geometry and charge partition [16]. One sulfate ion binding site is the same as that of the phosphate, the other one being located near the hinge. The mechanism by which the native structure of PGK is stabilized by phosphate perhaps includes several contributions, a specific binding to the γ- or β-phosphate site of nucleotides and a bulky charge effect.

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